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ORIGINAL CONTRIBUTION

Differentiation between *Aphis pomi* and *Aphis spiraecola* using multiplex real-time PCR based on DNA barcode sequences

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Abstract

The green apple aphid (*Aphis pomi)* and the spirea aphid (*Aphis spiraecola*) are pests of apples in North America. Although management regimes exist to effectively control these pests, they differ significantly because of varying susceptibility of each species to common pesticides and differences in their life cycles. Therefore, accurate identification of the species present is essential for pest control. However, the identification process is complicated because of the morphological similarity between these two species. As a result, confusion between *A. pomi* and *A. spiraecola* often occurs. DNA barcoding has been proven to accurately identify species of Aphididae. A further study demonstrated that DNA barcodes could be used to accurately differentiate *A. pomi* and *A. spiraecola*. DNA barcoding represents an important step towards rapid identification of these pests as distinctions can be easily made between morphologically similar species as well as from eggs and immature individuals in addition to adults. However, samples must still be sent to specially equipped facilities for sequence analysis, which can take between several hours and days. Real-time PCR is emerging as a useful tool for more rapid pest identification. The purpose of this study was to develop a real-time PCR assay for differentiation of *A.pomi* from *A. spiraecola* based on DNA barcode sequences from the Barcode of Life Data System. This assay was designed on the portable SmartCycler II platform and can be used in field settings to differentiate these species quickly and accurately. It has the potential to be a valuable tool to improve pest management of *A. pomi* and *A. spiraecola*.

Introduction

The green apple aphid (*Aphis pomi* De Geer 1783) and the spirea aphid (*Aphis spiraecola* Patch 1914) are pests of apples in North America (Foottit et al. 2006). They play a role in disease transmission (Blackman and Eastop 2000) and affect healthy growth of their hosts (Kaakeh et al. 1993). Although management regimes exist to effectively control these pests, they differ significantly because of varying susceptibility of each species to common pesticides (Lowery et al. 2005, 2006) and differences in their life cycles (Foottit et al. 2009). Therefore, accurate identification of the species present is essential for pest control. However, the identification process is complicated because of the morphological similarity between these two species. As a result, confusion between *A. pomi* and *A. spiraecola* often occurs (Blackman and Eastop 2000; Foottit et al. 2009).

DNA barcoding using the 5′ end of the mitochondrial cytochrome C oxidase I (COI) gene (Hebert et al. 2003) has been proven to accurately identify most species of Aphididae (Foottit et al. 2008; Lee et al. 2011). A further study demonstrated that DNA

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barcodes could be used to accurately differentiate between *A. pomi* and *A. spiraecola* (Foottit et al. 2009). DNA barcoding represents an important step towards rapid identification of these pests as distinctions can be easily made between morphologically similar species as well as from eggs and immature individuals in addition to adults based on sequence differences as demonstrated recently in cereal aphids (Shufran and Puterka 2011). However, samples must still be sent to specially equipped facilities for sequence analysis, which can take between several hours and days.

Real-time PCR is emerging as a reliable method for pest insect identification (Huang et al. 2010; Walsh et al. 2005; Yu et al. 2005; Madani et al. 2005). These assays can produce results much quicker than traditional barcoding, often in 1 h, and the results are simple to interpret. The one-step process not only reduces the length of time required to identify specimens, but also reduces the chance of contamination by eliminating the post-PCR processing steps necessary for DNA barcoding. In addition to being more rapid than DNA barcoding, some real-time PCR platforms are portable. Paired with the use of lyophilized reagents, this would allow accurate identification in field settings, eliminating the need to send samples off-site.

Species-specific primers and probes for use with real-time PCR can be designed using the DNA barcode sequences of target species in publically available projects in the Barcode of Life Data System (BOLD; http://www.boldsystems.org; Ratnasingham and Hebert 2007). Using sequences from related individuals to avoid cross-amplification with other species is an important aspect of primer and probe design. As errors arising from misidentification are often found in GenBank (Harris 2003; Vigalys 2003), the use of sequences from vouchered specimens in BOLD can lead to more accurate primer and probe design. The objective of this study was to create a real-time PCR assay for accurate and rapid differentiation of *A. pomi* and *A. spiraecola*, based on COI DNA barcode sequences from BOLD, to be run on a portable platform.

Material and Methods

Multiplex PCR Primer and probe design

Species-specific primers and TaqMan probes were developed for *Aphis pomi* and *Aphis spiraecola* using 690 COI barcode sequences from 338 species in the publically available BOLD project 'Barcoding the Aphididae'. These sequences were derived from individuals collected over a wide geographical range. Primer and probe design was based on all unique haplotypes from this project. Haplotype analysis was carried out using the haplotype variation tool at ibarcode.org. In total, 209 unique haplotypes were used for primer and probe design, including a single haplotype of *A. pomi* from four individuals collected from Canada and the US and a single haplotype of *A. spiraecola* from 11 individuals collected from Canada, the United States, Palau, Marshall Islands, Hawaii and Guam. Primers and probes were also tested for specificity against sequences from unique haplotypes of *A. spiraecola* from the public BOLD project 'Aphis Species on Apple (*A. pomi, A. spiraecola*)'. These haplotypes were identical to the haplotypes used in primer and probe design at the primer and probe sites. Primers and probes (Table 1) were developed using AlleleID (version 7.7; Premier Biosoft International, Palo Alto, CA, USA). The *A. pomi* -specific probe was tagged at the 5′ end with the fluorescent reporter tetrachloro-6-carboxyfluorescein (TET) and at the 3′ end with Black Hole Quencher-1 (BHQ-1) quenching dye. The *A. spiraecola* -specific probe was tagged with the fluorescent reporter 6-carboxyfluorescein (FAM) at the 5′ end, and BHQ-1 at the 3′ end. Optimization of probe and primer concentrations and cycling conditions was carried out according to the guidelines set by Cepheid for the Smart Cycler II System (SmartNote 6.2; http://www.cepheid.com/systems-and-software/smartcycler-system/).

Species selection and sample collection

A number of species with various degrees of relatedness to the target species were selected for specificity testing, following the classification scheme of

Table 1 Sequences of species-specific oligonucleotides used in this study and respective amplicon length. All primers and probes target portions of the C oxidase I DNA barcode region

	Aphis pomi	*Aphis spiraecola*
Forward primer sequence (5′-3′)	TGCCCAGATATA TCTTTCC	GAGCAATTAATTTT ATTTGTACA
Reverse primer sequence (5′-3′)	CCTGTTCCT GTTCCATTA	GCTAGAACTGGTAGAGA
Probe sequence (5′-3′)	TET-AGATTCTGATTATT ACCGCCTTCACT-BHQ1	6FAM-TTAAATCAAAT CCCACTATTTCCATG-BHQ1
Amplicon Length	110 base pairs	130 base pairs

Remaudière and Remaudière (1997). The following species were selected: other species of genus *Aphis* (*Aphis coreopsidis* (Thomas), *Aphis craccivora* Koch, *Aphis fabae* Scopoli, *Aphis farinose* Gmelin, *Aphis glycines* Matsumura, *Aphis gossypii* Glover, *Aphis helianthi* Monell in Riley and Monell, *Aphis hyperici* Monell in Riley and Monell, *Aphis illinoisensis* Shimer, *Aphis impatientis* Thomas, *Aphis lugentis* Williams, *Aphis maculatae* Oestlund, *Aphis nasturtii* Kaltenbach, *Aphis neilliae* Oestlund, *Aphis neogillettei* Palmer, *Aphis nerii* Boyer de Fonscolombe, *Aphis oestlund Gillettei, Aphis rubicola* Oestlund and *Aphis varians* Patch; other members of tribe Aphidini (*Braggia eriogoni* (Cowen ex Gillette and Baker) and *Sanbornia juniperi* Pergande ex Baker); representatives of the related tribe *Macrosiphini* of subfamily Aphidinae (*Acyrthosiphon pisum* (Harris), *Aulacorthum solani* (Kaltenbach), *Lipaphis pseudobrassicae* (Davis), *Macrosiphum rosae* (Linnaeus), *Myzus ascalonicus* Doncaster, *Myzus persicae* (Sulzer), and *Sitobion avenae* (Fabricius)); and a representative of the distantly related subfamily Eriosomatinae (*Eriosoma lanigerum* (Hausmann) that shares the same host plant as the target species). All collections were made into 95% ethanol. Detailed collection data are given in Table 2. Voucher specimens for these collections were deposited in the Canadian National Collection of Insects (Agriculture and Agri-Food Canada, Ottawa).

DNA extraction

DNA extraction was carried out on individual aphids using the DNeasy Blood and Tissue kit (Qiagen, Mississauga, Ontario, Canada) according to manufacturer guidelines with the following exception: elutions were performed in 100 μl of elution buffer and passed through the extraction column twice. Concentrations were determined using a Nanodrop 8000 (Thermo Scientific, Wilmington, DE, USA) and ranged from 4.28 to 112.3 ng/μl depending on the species of aphid.

Real-time multiplex PCR

Primer and probe sets for *A. pomi* and *A. spiraecola* (Integrated DNA Technologies, Coralville, IA, USA) were combined in a multiplex assay. Reactions were carried out in 25 μl volumes containing sterile water, 5 μl template DNA, each forward primer at a concentration of 0.3 μM, each reverse primer at a concentration of 0.5 μM and each probe at a concentration of 0.1 μM. OmniMix HS lyophilized master mix (Cepheid, Sunnyvale, CA, USA) was used according to manufacturer guidelines. PCR cycling conditions were as follows: an initial step of 95°C for 120 s, followed by 40 cycles of 94°C for 11 s, 60°C for 40 s and 72°C for 25 s. Fluorescence readings were taken at the annealing step of each cycle. All reactions were carried out on the SmartCycler II platform (Cepheid). The cycle threshold (C_t) value was determined as the number of cycles at which the fluorescence reading exceeded the detection threshold. If no signal was observed, a C_t value of 40 was assigned. The multiplexed set was tested using DNA from 10 individuals of each of the two target species and DNA from three individuals of each of the species selected for background testing. To determine the ability of the multiplex assay to detect both species simultaneously, DNA admixtures containing the following percentages for each species were tested: 50% *A. pomi* and 50% *A. spiraecola*, 80% *A. pomi* and 20% *A. spiraecola*, 90% *A. pomi* and 10% *A. spiraecola*, 20% *A. pomi* and 80% *A. spiraecola* and 10% *A. pomi* and 90% *A. spiraecola*.

Sensitivity and linearity

Standard curves were generated from 10-fold serial dilutions of *A. pomi* DNA and *A. spiraecola* DNA from 10 ng/μl to 1 pg/μl to determine the linear range, detection limits and efficiency of the multiplex assay.

Results

The FAM fluorescence channel showed a signal for *A. spiraecola* between C_t = 17.21 and 19.7 and the TET fluorescence channel showed a signal for *A. pomi* between C_t = 17.66 and 21.16. Signals were observed in either fluorescence channel for only three of the 29 non-target species: in the TET fluorescence channel, a signal for *Aphis rubicola* between C_t = 26.66 and 40 was observed and in the FAM fluorescence channel, signals were observed between C_t = 36.86 and 40 for *Aphis neogillettei* and between C_t = 38.45 and 40 for *Aphis coreopsidis*. No false negatives were observed in this study, and no signal was observed for any no-template controls. The assay was also proven to successfully detect both species in DNA admixtures with as low as 10% of the DNA belonging to one of the two targets (Table 3). Standard curves of the TET and FAM fluorescence channels for the multiplexed primer and probe set showed a linear range from 10 ng/μl to 1 pg/μl for both target species, with a detection limit of 1 pg/μl (fig 1). Efficiencies were over 99% for each fluorescence

Table 2 Collection of information for aphid specimens used in this study

Species	Sample identifier	Locality	Latitude	Longitude	Date	Host
Aphidinae: Aphidini						
Aphis coreopsidis (Thomas)	CNC#HEM049398	USA: Georgia: Sirman	31.100	−82.961	2002/06/04	*Baccharis halimifolia*
Aphis craccivora Koch	CNC#HEM050467	Guam: Barrigada	13.509	144.832	2003/02/26	*Spondias purpurea*
Aphis fabae Scopoli	CNC#HEM057623	Canada: British Columbia: Sutton Pass	49.280	−125.355	2007/08/22	*Rumex* sp.
Aphis farinosa J.F. Gmelin	CNC#HEM057573	Canada: British Columbia: Kitamaat Village	53.981	−128.650	2007/08/18	*Salix discolour*
Aphis glycines Matsumura	CNC#HEM052292	Canada: Quebec: Sainte-Anne-des-Plaines	45.775	−73.828	2004/07/21	*Glycine max*
Aphis gossypii Glover	CNC#HEM007420	Canada: Ontario: Wolf Island	44.208	−76.259	1993/05/22	*Hibiscus* sp.
Aphis helianthi Monell	CNC#HEM057205	Canada: Manitoba: Burntwood River	55.755	−97.843	2007/07/21	*Cicuta* sp.
Aphis hyperici Monell	CNC#HEM049278	USA: North Carolina: Rainbow Springs	35.093	−83.556	2002/05/30	*Hypericum prolificum*
Aphis illinoisensis Shimer	CNC#HEM049338	USA: Tennessee: Tallassee	35.552	−84.091	2002/06/01	*Vitis* sp.
Aphis impatientis Thomas	CNC#HEM070495	Canada: Ontario: Ottawa	45.386	−75.704	2010/08/31	*Impatiens capensis*
Aphis lugentis Williams	CNC#HEM049241	USA: North Carolina: Buck Creek	35.086	−83.616	2002/05/30	*Senecio plattensis*
Aphis maculatae Oestlund	CNC#HEM057479	Canada: Ontario: Prairie Bee River	47.863	−83.906	2007/07/30	*Populus tremuloides*
Aphis nasturtii Kaltenbach	CNC#HEM007709	Canada: Ontario: Packenham	45.341	−76.291	1994/06/05	*Rhamnus cathartica*
Aphis neilliae Oestlund	CNC#HEM070027	Canada: Ontario: Pitopiko River	49.769	−84.773	2010/07/09	*Physocarpus opulifolius*
Aphis neogillettei Palmer	CNC#HEM070051	Canada: Ontario: Dryden	49.786	−92.810	2010/07/10	*Cornus stolonifera*
Aphis nerii Boyer de Fonscolombe	CNC#HEM009682	Canada: Ontario: Ottawa	45.377	−75.655	1994/10/08	*Asclepias syriaca*
Aphis oestlundi Gillette	CNC#HEM070326	Canada: Ontario: Ottawa	45.371	−75.733	2010/08/20	*Oenothera biennis*
Aphis pomi DeGeer	CNC#HEM039309	Canada: Ontario: Yonge Mills	44.506	−75.835	2001/07/12	*Malus domestica*
Aphis rubicola Oestlund	CNC#HEM070500	Canada: Ontario: Ottawa	45.386	−75.704	2010/08/31	*Rubus idaeus* ssp. *strigosus*
Aphis spiraecola Patch	CNC#HEM033926	Canada: British Columbia: Penticton	49.473	−119.583	2000/05/27	*Spiraea* sp.(ornamental)
Aphis varians Patch	CNC#HEM057244	Canada: Manitoba: Odei River	55.994	−97.357	2007/07/22	*Epilobium angustifolium*
Braggia eriogoni Cowen	CNC#HEM033259	Canada: British Columbia: Williams Lake	51.973	−122.666	2000/07/20	*Eriogonum heracloides*
Sanbornia juniperi Pergande	CNC#HEM069879	USA: Colorado: Drake	40.450	−105.386	2010/06/23	*Juniperus scopularum*
Aphidinae: Macrosiphini						
Acyrthosiphon pisum (Harris)	CNC#HEM070514	Canada: Ontario: Ottawa	45.033	−75.902	2010/09/05	*Coronilla varia*
Aulacorthum solani (Kaltenbach)	CNC#HEM056959	Canada: Ontario: Malakoff	45.111	−75.788	2007/07/10	*Thalictrum* sp.
Lipaphis pseudobrassicae (Davis)	CNC#HEM070511	Canada: Ontario: Ottawa	45.033	−75.902	2010/09/04	*Erucastrum gallicum*
Macrosiphum rosae (Linnaeus)	CNC#HEM070223	Canada: Ontario: Ottawa	45.389	−75.708	2010/08/10	*Rosa* sp.
Myzus ascalonicus Donscaster	CNC#HEM061938	Canada: British Columbia: Langley	49.100	−122.600	2008/05/07	*Agoseris aurantiaca*
Myzus persicae (Sulzer)	KSP 24	USA: Washington: Prosser	46.257	−119.729		*Solanum tuberosum*
Sitobion avenae (Fabricius)	CEF colony	Canada: Manitoba: Winnipeg	49.883	−97.150	1995/08/15	Poaceae
Eriosomatinae						
Eriosoma lanigerum Hausmann	CNC#HEM007886	Canada: Nova Scotia: Wentworth	45.633	−63.432	1994/08/07	*Malus domestica*

Table 3 Demonstration of simultaneous detection of *Aphis pomi* (TET) and *Aphis spiraecola* fluorescent reporter 6-carboxyfluorescein (FAM) from DNA admixtures containing varying percentages of DNA from each species. The average Ct values recorded from each target fluorescence channel in a series of multiplexed runs are shown

% *A. pomi* DNA	% *A. spiraecola* DNA	Average TET C_t	Average FAM C_t
100	0	17.9	40
0	100	40	18.7
50	50	18.93	18.98
80	20	18.37	19.87
90	10	18.17	21.75
20	80	20.99	18.3
10	90	21.69	17.99

channel in the multiplex assay, and r^2 values were 0.999.

Discussion

Using aphid sequences available in BOLD, a real-time multiplex PCR assay was designed that successfully differentiated *Aphis pomi* from *Aphis spiraecola*. Overall the assay was specific to the target species.

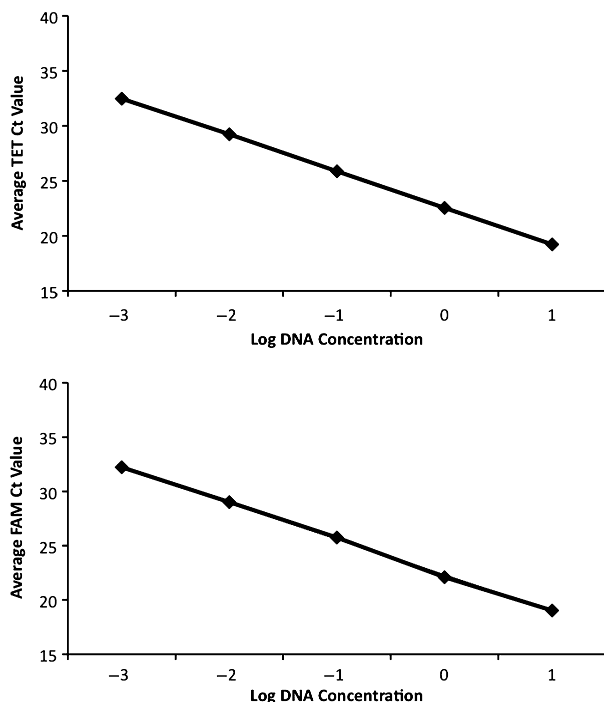

Fig. 1 Linearity of multiplex assay for *Aphis pomi* (TET; $r^2 = 0.999$, slope = −3.319) and *Aphis spiraecola* (fluorescent reporter 6-carboxyfluorescein (FAM); $r^2 = 0.999$, slope = −3.331). Standard curves are generated from 10-fold serial dilutions of *A. pomi* or *A. spiraecola* DNA from 10 ng/μl to 1 pg/μl.

Although some cross-amplification was observed with a small percentage of non-target species, these were largely delayed ($C_t > 35$). Only one species of the 29 tested gave a signal under $C_t = 35$ and therefore could cause a false positive in routine analysis. The use of a cutoff value of $C_t = 25$ to indicate a positive result from target species would circumvent this issue, as the highest observed C_t value for *Aphis pomi* was 21.16 and the lowest observed C_t value for *Aphis rubicola* was 26.66. The suitability of this cutoff could be confirmed with an analysis of more individuals of *Aphis rubicola* during validation of this assay. A C_t cutoff value of 25 is unlikely to cause confusion between a false-positive and a weak signal due to low DNA concentration of the target, as this value corresponds to a DNA concentration of the target species of <0.1 pg/μl, which is over 40 times less concentrated than the lowest concentration obtained from DNA extraction from a single aphid observed in this study. Furthermore, in practice, this deficiency is not likely to be a problem because such a test is likely to be applied only in situations in which aphid species that are likely to give a false-positive result do not occur. For example, in the current context, only other aphid species that are likely to be found on apple need be considered.

For the target species, a 100% correlation between the morphologically assigned species and the species identified using real-time PCR was observed, indicating the method could replace or confirm morphological identification of adults, with the added benefit of being applicable to egg and immature forms. The wide range of DNA concentrations used in this study did not appear to impact the ability to identify the target species from single individuals.

It was possible to detect both species simultaneously, suggesting that extraction of multiple aphids at once will not affect the ability to detect one or both of these species in the resulting mixture, reducing the cost per individual aphid identification.

Although the PCR protocol takes under 1 h, the lysis step in the extraction protocol adds time to the identification process. However, this processing time could be greatly reduced with the adoption of more rapid extraction methods (e.g. Hunter et al. 2008; Castalanelli et al. 2010) to obtain results much more quickly. With rapid identification available, appropriate pest management regimes can be put into place almost immediately. The use of the portable Smart Cycler II system makes on-site analysis possible. Because of the simplicity of assay and the ease with which results can be interpreted, after limited training, the identification of specimens can be carried

out by non-specialists while retaining high confidence in the results. Lyophilizing all reagents in the appropriate concentrations for sale in commercial kits would even further reduce the likelihood of human error.

All known haplotypes of the target species, as well as sequences from other aphid species known to feed on apples in North America, were used in the *in silico* design of primers and probes published here. However, it will be important for future studies to focus on the practical confirmation of the specificity of these primers and probes. Although further optimization and validation of these results is necessary, the data presented here show that this assay is an effective approach to complement current methods of differentiating between *A. pomi* and *A. spiraecola*. This study also confirms the utility of DNA barcode sequences as a resource for designing probes and primers for real-time PCR assays to distinguish between morphologically similar species. As BOLD continues to grow, and more pest species and their close relatives are referenced, comparable assays could be designed to do the same for other morphologically similar pairs or small groups.

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